Decreased miR-340 expression in bone marrow is associated with liver metastasis of colorectal cancer

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Keywords: miR-340, DTC (disseminated tumor cell), colorectal cancer, bone marrow, liver metastasis.


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Disclosure of Potential Conflicts of Interest

The authors have declared that they have no competing interests.
Abstract

Studies have shown the prognostic significance of disseminated tumor cells (DTCs) in bone marrow (BM) of patients with colorectal cancer (CRC). However, the molecular characteristics of DTCs, including their miRNA expression profiles, remain mostly unknown. In this study, we analyzed the miRNA expression of DTCs in BM. EpCAM⁺ BM cells were collected using immunomagnetic beads after exclusion of CD14⁺ and CD45⁺ cells, then subjected to miRNA microarray analysis. Cluster analysis (7 CRC patients with liver metastasis and 12 CRC patients without liver metastasis) indicated that miR-340 and miR-542-3p expressions were significantly decreased in EpCAM⁺ BM cells of patients with liver metastasis (P = 0.019 and 0.037, respectively). We demonstrated that pre-miR-340 administration inhibited growth of colon cancer cells and suppressed c-Met expression in vitro. In clinical samples of CRC, miR-340 was expressed at significantly lower levels in tumor tissues compared to normal mucosa. Survival analysis in 136 CRC patients indicated that low miR-340 expression was correlated with shorter 5-year disease-free survival (P = 0.023) and poor 5-year overall survival (P = 0.046). It was of note that the CRC group with low miR-340 and high c-Met expression had the worst prognosis. We further demonstrated that systemic pre-miR-340 administration suppressed growth of pre-established HCT116 tumors in animal therapeutic models. These findings indicate that miR-340 may be useful as a novel prognostic factor and as a therapeutic tool against colorectal cancer. Our data
suggest that miR-340 in bone marrow may play an important role in regulating the metastasis cascade of colorectal cancer.
Introduction

Disseminated tumor cells (DTCs) are thought to represent the beginning of systemic disease from localized human cancer. In patients with various types of tumors, DTCs are detectable in regional lymph nodes, peripheral blood, and bone marrow (BM) at early stages of tumor progression (1, 2). Cumulative evidence indicates that metastatic relapse is significantly correlated with the presence of DTCs in BM in several tumor types, including carcinomas of the colorectum (3, 4), breast (5, 6), lung (7, 8), and prostate (9, 10). These findings suggest that DTCs in BM can be a sensitive marker for cancer spread from the primary tumor, although they are usually present at a microscopic level. Two methods are commonly employed to detect DTCs in BM: immunological assays using antibodies directed against specific epithelial cell proteins, such as cytokeratins, EpCAM, and Ber-EP4 (11); and PCR-based molecular assays targeting CK19, CK20, EpCAM, and CEA transcripts (12).

Many studies have reported that DTCs in BM are associated with prognosis; however, little is known about how DTCs acquire metastatic properties. Previous reports indicate that all patients with DTCs do not necessarily develop distant metastasis (3, 5, 10). Therefore, it is likely that the DTCs of different individual case have varying characteristics that influence their ability to develop metastasis. It was recently reported that the VEGFR-1 or miR-144-ZFX axis plays a role in the BM-associated metastasis cascade of gastric cancer (13, 14).
miRNAs - small noncoding RNA gene products ranging in size from 19 to 25 nucleotides - have been identified as factors involved in cancer development and metastasis through negative regulation of oncogenic or anti-oncogenic genes (15). miRNAs can play roles in regulating mRNA translation and degradation through base pairing to partially complementary sites, predominantly in the untranslated region of the mRNA (15). Through this negative regulation of gene expression, miRNAs can influence various biological processes, including cell proliferation, cell death, and stress resistance (16).

To test the hypothesis that DTCs in BM might have a specific miRNA expression profile when they acquire the metastatic potential, here we collected BM samples from CRC patients with liver metastasis and non-metastatic patients, and analyzed the miRNA profile differences between the two groups. Our data provide novel information and help further our understanding of the BM-mediated metastasis cascade of CRC.
Material and Methods

Clinical samples

BM samples were collected from 19 colorectal cancer (CRC) patients (7 with liver metastasis and 12 without metastasis) during surgery at Osaka University Hospital between 2010 and 2012. Supplementary Table S1 shows the characteristics of these 19 patients. Among the cases with liver metastasis, five cases were synchronous (including three patients with multiple liver metastases and two with a single liver metastasis) and two were metachronous (one with multiple liver metastases and one patient with a single liver metastasis).

Additionally, 136 primary CRC samples were collected from patients who had curative surgery between 1999 and 2010 at Osaka University Hospital and its two related hospitals. The tumor samples were stored at −80°C with RNAlater® until RNA extraction. These 136 patients included 4 stage 0, 15 stage I, 38 stage II, 52 stage IIIA/B, and 27 stage IIIC CRC diseases. The mean follow-up period for these patients was 4.2 years. All patients gave written informed consent, in accordance with the guidelines approved by the Institutional Research Board of each institute. This study was conducted under the supervision of the ethical board of Osaka University Hospital.

Bone marrow aspiration and MACS sorting
CRC patients were administered anesthesia and 20 mL BM was taken from the right and left anterior iliac crests before surgery. Mononucleated cells were collected using a standard Ficoll-Hypaque gradient technique. To enrich for EpCAM⁺ cells, CD14⁺ cells were removed (designated as BM2) from the whole bone marrow (BM1, Fig. 1A) using auto MACSTM pro (Milteny Biotec, Bergisch Gladbach, Germany) with anti-CD14 immunomagnetic beads (clone; TÜK4, Milteny Biotec). Next, CD45⁺ cells were removed by treatment with anti-CD45 immunomagnetic beads (clone; 5B1; Milteny Biotec), and the CD14⁻CD45⁺ cells were designated as BM3. The residual CD14⁻CD45⁻ cells were then incubated with FcR blocking reagent (Milteny Biotec), followed by incubation with anti-EpCAM immunomagnetic beads (clone; HEA-125, Milteny Biotec), and the CD14⁻CD45⁻EpCAM⁺ cells were taken up and designated BM4 (Fig. 1A).

**Immunocytochemistry**

Cells were attached onto glass slides and quickly air-dried. After permeabilization with 70% ethanol for 30 min, immunocytochemistry with anti-cytokeratin-large spectrum antibody (clone; KL1, Beckman Coulter, Fullerton, CA) at a 1:1000 dilution was performed using a standard ABC method as previously described (17, 18). Fluorescent immunochemistry was performed using FITC-conjugated anti-human epithelial antigen antibodies (clone; Ber-EP4, DAKO, Glostrup, Denmark) at a 1:100 dilution and observed with a BZ-9000 fluorescence microscope (Keyence, Osaka,
RNA isolation and miRNA microarray analysis

Total RNA was isolated using the miRNeasy kit (Applied Biosystems, Carlsbad, CA) following the manufacturer’s protocol. Total RNA concentration and purity were assessed with a spectrophotometer, and RNA integrity was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Total RNA (100 ng) was directly labeled with cyanine 3-CTP (Cy3), without fractionation or amplification, using an Agilent protocol that produces precise and accurate measurements spanning a linear dynamic range from 0.2 amol to 2 fmol of input miRNA. Each 100 ng total RNA sample was competitively hybridized to a miRNA array (Agilent Microarray Kit; G4470C) containing 866 miRNAs (version 12.0 of the Sanger miRNA database; http://www.mirbase.org/) (19). The intensity of each hybridization signal was evaluated using Feature Extraction Software Version 10.7.3.1 (Agilent Technologies), which used the global normalization method (90percentile shift) using GeneSpring GX Software Version 11.5.1 (20). P values were calculated using an unpaired t-test. All data from the miRNA array have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database with accession code GSE51716.

cDNA microarray
Total RNA (500 ng) was converted into labeled cRNA with Cy3-coupled nucleotides (PerkinElmer) using the Quick Amp Labeling Kit, one-color (Agilent Technologies). Cy3-labeled cRNA (1.65 mg) was hybridized to an oligonucleotide microarray (Whole Human Genome 4x44 K Agilent G4112F) for 17 hours at 65°C. The intensity of each hybridization signal was evaluated using Feature Extraction Software Version 10.7.3.1 (Agilent Technologies). \( P \) values were calculated using an unpaired \( t \)-test.

**Reverse transcription PCR and TaqMan miRNA assay**

The TaqMan miRNA Assay (Applied Biosystems) was used to measure miRNA levels. First, 5 ng RNA was reverse transcribed, and the resulting cDNA was amplified using the following specific TaqMan miRNA assays: hsa-miR-340 ID 002258, hsa-miR-542-3p ID 001284, and RNU6B ID 001093. The qRT-PCR reactions were performed using the 7900HT Sequence Detection System (Applied Biosystems), following the manufacturer’s protocol. Amplification data were normalized to RNU6B expression. Relative expression was quantified using the \( 2^{-\Delta\Delta Ct} \) method.

**Reverse transcription mRNA PCR and quantitative real-time PCR**

The LightCycler TaqMan® Master (Roche Diagnostics, Basel, Switzerland) was used to measure mRNA levels. First, 500 ng total RNA was reverse transcribed
using High Capacity RNA-to-DNA™ Kit (Applied Biosystems), and the resulting cDNA was amplified using the following specific primers: c-Met: forward primer, 5’-AAATGTGATGAAGCAGGAA-3’, reverse primer, 5’-TCTCTGAAATTAGAGCGA TGTTGA-3’, MITF: forward primer, 5’-CAGCGTGTATTTTTCCCACA-3’, reverse primer, 5’-TGCGGTCATTTATGTTAAATCTTC, Survivin: forward primer, 5’-GCCC AGTGGTTTCTTCTGCTT-3’, reverse primer, 5’-AACCGGACGAATGCTTTTTA-3’, ACTB: 5’-AGAGCTACGAGCTGCCTGAC-3’, reverse primer, 5’-CGTGGATGCGCA CAGGACT-3’.

The qRT-PCR reactions were performed using the LightCycler® 2.0 System (Roche Diagnostics), following the manufacturer’s protocol. Amplification data were normalized to beta actin (ACTB) expression.

**Cell lines and culture**

Human CRC cell lines HCT116 and SW480 were obtained from the American Type Culture Collection (Rockville, Maryland) in 2001. Stocks were prepared after passage 2 and stored in liquid nitrogen. All experiments were performed with cells of passage of <8. These cell lines were authenticated by morphologic inspection, short tandem repeat profiling, and Mycoplasma testing by ATCC. Mycoplasma testing was done also by the authors. They were cultured in Dulbecco’s modified Eagle’s medium
(DMEM) containing 10% fetal bovine serum and 1% antibiotic/antimycotic solution (Sigma, St. Louis, MO) at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

**Transient miRNA transfection**

Cells were transfected with 30 nmol/L pre-miR miRNA precursor molecules of hsa-miR-340 and has-miR-542-3p (Applied Biosystems) using Lipofectamine iMax (Invitrogen, Darmstadt, Germany) in 6 or 12-well plates following the manufacturer’s protocol. Pre-miR negative control (Applied Biosystems) was used as a control.

**Proliferation assays**

Cells were seeded at a density of 3 - 4 × 10⁴ cells/well in 12-well dishes, and cultured for 72 hours to determine proliferation. Cell counting was performed using a NucleoCounter kit (Chemometec, Gydevang 43, DK-3450 Alleroed, Denmark).

**In vivo tumor growth**

HCT116 cells were mixed with Matrigel (BD Biosciences, San Jose, CA) and medium in a 1:1 ratio by volume. Cells (1 × 10⁶) in 100 μL of medium/Matrigel solution were injected s.c. into the bilateral lower back region of female nude mice (NIHON CLEA, Tokyo, Japan). Formulated miRNA (40% conjugation (21); 30 μg per injection) was administered intravenously with carbonate apatite as vehicle (22, 23) via tail vein
injections when tumor volume reached ~75 to 80 mm$^3$. Mice were treated four times with formulated miR-340 ($n = 7$) or miR-NC ($n = 9$), every 3 days. mature hsa-miR-340 sense (5′-UUAAUAAGCAUGAGACUGAUU-3′) and antisense (5′-AAUCAGUCUAUGCUUUUAUAA-3′), and negative control sense (5′-UAAAUGUACUGCGUGAGGAGGAA-3′) and antisense (5′-UUCCUCUCCACGCAGUACAUUU-3′) were purchased from Gene Design (Osaka, Japan). Tumor volumes were determined as previously described (22). All animal experiments were performed in accordance with the currently prescribed guidelines and using a protocol approved by Osaka University.

**Data analysis and statistics**

Statistical analysis was performed using the JMP9 program (SAS Institute, Cary, NC). Clinicopathologic factors were compared using the chi-square test and continuous variables were compared using Student’s $t$-test. Survival curves were computed using the Kaplan–Meier method and Cox hazard model, and statistically significant differences between survival curves were determined using the log-rank test and Cox hazard ratio. *In vivo* and *in vitro* analysis, data are expressed as mean ± SE and were analyzed with the Mann-Whitney U test. $P$-values < 0.05 were considered statistically significant.
Results

Characteristics of CD14−CD45−EpCAM+ cells

To obtain the EpCAM+ fraction, CD14+ cells and CD45+ cells were excluded from the whole bone marrow (BM1), then EpCAM+ cells were collected using the anti-EpCAM (HEA-125) antibody and the CD14−CD45−EpCAM+ fraction was named BM4 (Fig. 1A). We first compared gene expression between BM4 cells and the CD14+ BM2 cells, which were expected to be of monocyte or macrophage lineage and to thus not include epithelial cells. Compared to BM2, BM4 showed 9.25-fold higher levels of the epithelial marker E-cadherin mRNA, a 0.48-fold change in the mesenchymal marker Vimentin mRNA, and no change in N-cadherin level (Fig. 1B). Furthermore, BM4 exhibited significantly increased levels of the putative tumor marker CEA mRNA and the oncogene MYC mRNA (19.5-fold and 2.26-fold, respectively) (Fig. 1C). Immunostaining of BM4 cells using the epithelial cell specific antibodies Ber-EP4 Ab (Fig. 1D) and cytokeratin Ab (Fig. 1E) revealed the presence of tumor cell clusters, comprising individual cells of > 10 μm in diameter.

Distinct miRNA profiles in BM4 cells between liver metastasis and non-metastasis

Using the miRNA microarray, we analyzed the miRNA levels of BM4 cells obtained from non-metastasis patients (n = 12) and liver metastasis patients (n = 7). Cluster analysis revealed 10 miRNAs that showed a >1.50-fold change (range, 1.50-
3.39-fold) with significant between-group differences ($P < 0.05$) (Fig. 1F). Five miRNAs were decreased and the other five were increased in BM4 cells from the CRC patients with liver metastasis compared to in those without metastasis. Table 1 presents information about each miRNA, including the target molecule and whether it is an anti-oncomir or oncomir according to the available literature (24-37). All data from the miRNA array are shown in Supplementary microarray results.

### Validation of miRNA expression by qRT-PCR

We performed qRT-PCR assays to verify the significant differences for the two miRNAs that were most downregulated in patients with liver metastasis: miR-340 and miR-542-3p. Our results confirmed that compared to in non-metastasis patients, liver metastasis patients showed significantly lower BM4 cell expressions of miR-340 and miR-542-3p ($P = 0.008$ and $0.015$, respectively; Supplementary Fig. 1).

### miR-340 and miR-542-3p inhibited CRC cell proliferation in vitro

In HCT116 and SW480, pre-miR-340 and pre-miR-542-3p treatment significantly inhibited cell growth compared to that in parental cells ($P < 0.001$) or in negative control miR-treated cultures ($P < 0.01$) (Fig. 2A and 2B). These in vitro proliferation assays were repeated three times and similar results were obtained. When we examined the levels of miRNAs in HCT116 and SW480 cells, both miR-340 and miR-542-3p levels were
significantly higher at 24 h as compared to those treated with pre-miR-negative control, and those in normal colonic mucosa ($P < 0.05$, Fig. 2A and 2B).

**Expression of miR-340 and miR-542-3p in normal colonic mucosa and CRC tissue samples**

Normal mucosa showed significantly higher miR-340 expression compared to in CRC tissue ($P = 0.010$; Fig. 2C). No significant difference was found in miR-542-3p expression ($P = 0.973$; Fig. 2C).

**Association between miR-340 and miR-542-3p expressions and CRC patient prognosis**

We analyzed miR-340 expression in tissue samples from 136 CRC patients who underwent curative surgery. Patients were divided into two groups: lower than average miR-340 expression ($n = 73$), and higher than average miR-340 expression ($n = 63$). Kaplan–Meier estimation indicated that high miR-340 expression was significantly associated with better 5-year disease-free survival (DFS) and better 5-year overall survival (OS) ($P = 0.023$ and 0.046, respectively; Fig. 3A). Similar survival analysis showed that miR-542-3p expression had no effect on either 5-DFS or 5-OS ($P = 0.429$ and 0.577, respectively; data not shown). Clinicopathological survey indicated that
miR-340 expression correlated with smaller tumor size \((P < 0.001)\) but not with any other factors (Supplementary Table 2).

Table 2 shows the results of univariate and multivariate analyses of factors related to 5-year DFS. Univariate analysis indicated that lymph node metastasis \((P = 0.003)\), serosal invasion \((P = 0.001)\), and low miR-340 expression \((P = 0.020)\) were significantly related to DFS. Multivariate analysis indicated that low miR-340 expression was an independent predictor of DFS [relative risk \((RR)\), 2.499; 95% confidence interval \((CI)\), 1.031–6.962; \(P = 0.042\)], as were serosal invasion \((RR, 3.053; 95\% CI, 1.311–7.041; \(P = 0.011\)) and lymph node metastasis \((RR, 2.918; 95\% CI, 1.081–10.16; \(P = 0.033\)) (Table 2).

**Systemic delivery of formulated miR-340 inhibited the growth of established CRC tumors in vivo**

In the therapeutic animal models of HCT116 tumors, tumor growth was significantly inhibited by systemic administration of formulated miR-340 compared to in the pre-miR negative control group \((P < 0.05)\) (Fig. 3B and 3C). This experiment was repeated twice, and reproducible results were obtained (data not shown). In order to evaluate the miRNAs delivery to the subcutaneous tumors, we measured the miR-340 level of the subcutaneous tumors at 1, 8, and 12 h after administration via tail vein. As shown in Fig. 3B, pre-miR-340 treated mice had significantly higher miR-340 levels at 1,
8, and 12h as compared to the control mice treated with pre-miR-negative control ($P < 0.01$ for each).

**Association between miR-340 and c-Met expressions and CRC patient prognosis**

We transiently transfected HCT116 and SW480 cells with pre-miR-340 and miR-542-3p, and then analyzed target gene expression, c-Met and MITF for miR-340, and Survivin for miR-542-3p. We observed that c-Met levels in pre-miR-340-treated HCT116 and SW480 cells were suppressed to 24.6 and 35.9% of that in negative control miR-treated cells, respectively (Fig. 4A). Similar results were obtained in terms of MITF and Survivin when HCT116 and SW480 cells were treated with pre-miR-340 and pre-miR-542-3p, respectively (Supplementary Fig. 2A and 2B).

We also confirmed c-Met expression level of the subcutaneous tumors at 8, 12, and 24 h *in vivo* models. The expression level of c-Met significantly decreased after systemic administration of pre-miR-340 compared to the pre-miR negative control group at each time point ($P < 0.01$) (Fig. 4B).

Furthermore, we evaluated the association between miR-340 and c-Met expression levels in 135 clinical samples, which was the part of the initial 136 clinical samples. There was not significant association between miR-340 and c-Met expression levels in the whole (data not shown). However, subgroup analysis revealed that
miR-340-low/c-Met-high cases had a significantly worse prognosis compared to miR-340-high/c-Met-low cases ($P = 0.015$; Fig. 4C)
Discussion

In 2002, a metastasis model was proposed that metastatic capacity was gained early during tumor development (38). In this model, BM is considered to be an important reservoir that allows DTCs to adapt, escape the host immune defenses in a dormant state, and develop into overt metastases in different organs (2, 39); however, the precise mechanism is largely unknown. In the present study, we attempted to uncover the nature of DTCs in BM of CRC patients who developed liver metastasis, from a view of miRNA expression.

Flatmark et al. (3) previously described the use of immunomagnetic selection with an anti-EpCAM antibody to determine the presence of DTCs in BM of CRC patients. EpCAM is a useful epithelial marker for detecting colorectal cancer cells, with reported detection rates ranging from 89.6–100% (40, 41). In the present study, we also employed an immunomagnetic capture method to encircle DTCs from BM aspirates using anti-EpCAM antibody (clone HEA-125), with some modifications. To facilitate DTC capture, CD14+ and CD45+ cells were removed beforehand. The resultant CD14−CD45−EpCAM+ fraction displayed typical DTC characteristics, i.e., high expressions of the epithelial marker E-cadherin, tumor-associated CEA, and oncogene MYC. DTC identity was further confirmed by microscopic observation with the BM4 fraction stained with EpCAM antibody (Ber-EP4 clone) and anti-cytokeratin antibody.
Thus, we verified that the employed method successfully enriched the BM4 fraction with DTCs from BM aspirates.

miRNA analysis of BM4 cells identified 10 miRNAs that were significantly differentially expressed between the non-metastasis and liver metastasis groups. It is notable that the putative oncomirs miR-222 (32, 42) and miR-155 (35-37) were increased in the BM4 cells of the liver metastasis group. Increased miR-155 expression has been reported in oral cell and pancreatic carcinomas (35, 37), and represents oncomir functions, including downregulation of anti-oncogenes, such as tumor protein 53-induced nuclear protein 1 (TP53INP1), suppressor of cytokine signaling 1 (SOCS1), and cell division cycle 73 (CDC73), which negatively regulates β-catenin, cyclin D1, and c-MYC (35-37).

On the other hand, miR-222 is increased in non-small lung cell and hepatocellular carcinoma, and it suppresses p27, phosphatase and tensin homolog (PTEN), and tissue inhibitor of metalloproteinases-3 (TIMP3), all of which are usually linked to tumor suppressive effects (32, 42). Literature survey revealed that 7 miRNAs included 4 anti-oncomirs, all of which were decreased in the liver metastasis group and 3 oncomirs, which exclusively increased in the metastasis group. The consistency between reported function of miRNAs and clinical outcome would justify collection method for DTCs and performance of employed miRNA array. These results also suggest that DTCs in BM may play a role in the establishment of liver metastasis through regulation of miRNA expression, i.e., downregulation of anti-oncomirs or upregulation of oncomirs.
Among the 10 miRNAs, we focused further experiments on those that were downregulated, since we expected they might have simple and physiological effects that could be useful in replacement therapy. Using qRT-PCR, we validated the results of miRNA array analysis for the two most strongly downregulated miRNAs: miR-340 and miR-542-3p. Although another cohort of bone marrow of CRC is expected to be investigated, further collection of 7-10 cases with liver metastasis and 10 cases without metastasis is practically difficult. For this reason, we attempted to investigate the malignant features of these miRNAs in CRC cell lines and clinical sample of the primary CRCs.

*In vitro* studies showed that addition of pre-miR-340 or pre-miR-542-3p led to inhibition of cell growth of colon cancer cells, suggesting these miRNAs to be anti-oncomirs, as has been reported in other cancer types: miR-340 in breast cancer (24) and colorectal (43), and miR-542-3p in colon (27) and lung cancer (26). Furthermore, pre-miR-340 and pre-miR-542-3p suppressed the target genes: c-Met (24) and MITF (25) for miR-340, and: Survivin (26) for miR-542-3p.

To further investigate the potential essential role of these anti-oncomirs, we examined their expression levels in clinical CRC samples, particularly in terms of normal versus tumor tissue, and the impact on patient survival. We found that expression of miR-340, but not miR-542-3p, was significantly decreased in tumor tissues compared to in normal mucosa. This finding supports the idea that miR-340 functions as an
anti-oncomir in CRC, and may be of clinical importance in replacement therapy against CRC. Moreover, multivariate analysis indicated that low expression of miR-340, but not miR-542-3p, was a significant predictor of poor 5-year DFS, and high miR-340 expression was significantly associated with smaller tumor size. These findings suggest that although both miR-340 and miR-542-3p act as anti-oncomirs in CRC cell lines \textit{in vitro}, only miR-340 may have clinical relevance.

Our finding was consistent with the previous report that the low expression of miR-340 was associated with poor prognosis in CRC (43). However, this study performed only the array analysis that included very small sample size (each group, n = 6) without further confirmation study using RT-PCR. In this regard, our data substantially extended the previous small study and have established a clinical value of miR-340 as a strong indicator for prognosis of CRC patients. In relation to disease relapse, liver metastasis is the most major recurrent mode of CRC. When we consider the link between miR-340 and liver metastasis, it is postulated that c-Met, proto-oncogene encoding a receptor tyrosine kinase might be a key molecule. A previous study demonstrated that c-Met gene amplification was linked to metastatic progression of CRC, especially in liver metastasis of CRC (44). On the other hand, a direct target of miR-340 is reportedly shown to be c-Met in breast cancer (24). It was of interest that combination analyses with miR-340 and c-Met showed that miR-340-low/c-Met-high group was solely a significant worse prognostic marker as compared to miR-340-high/c-Met-low group. These results
suggest that miR-340/c-Met axis may play an important role in metastasis cascade of CRC.

In animal models, it has been demonstrated that several anti-oncomir miRNA replacement therapies using either virus-mediated transduction or non-virus vehicle have inhibitory effects on cancer metastasis and tumor growth. Several examples include reduced tumor growth by adenoviral transfer of miR-let-7 in mouse models of lung cancer (45), and blockade of prostate cancer cell metastasis by miR-34a and miR-16 using RNA-Lancer II and atelocollagen, respectively (46, 47). Furthermore, anti-oncomir uptake is believed to confer no adverse effects to normal cells, because the pathway regulated by miRNA administration is already activated by the endogeneous miRNA (48). In the present study, we found that administration of miR-340 synthetic nucleotides via tail vein injection into nude mice significantly inhibited growth of pre-established HCT116 tumors. To our knowledge, this is the first evidence that systemic delivery of miR-340 exhibits anti-tumor effects in vivo in an animal therapeutic model. Since administration of synthesized miRNA agonist is considered clinically safer than virus-mediated techniques, miR-340 could be a promising therapeutic agent.

There is some unresolved debate regarding the exact contents of the BM4 fraction. It is obvious from our data that this EpCAM-positive fraction contains an enriched population of DTCs, as confirmed by immunostaining with the anti-Ber-EP4 antibody or the anti-CK antibody. However, we could not rule out a possibility that BM cells other
than DTC might express specific miRNAs. It has recently been proposed that niche cells surrounding tumor cells in the cancer microenvironment could cherish cancer cells and endow their metastatic ability (49, 50). This possibility is currently under investigation in our laboratory.

In conclusion, the results of the present study demonstrated that DTCs in BM of CRC patients with or without liver metastasis displayed distinct miRNA expression profiles, suggesting that the characteristics of DTCs, as defined by miRNA expression, may be essential for allowing tumor metastasis. Our data also imply that miR-340 is a novel prognostic factor, and could be a useful small nucleic acid for replacement therapy against CRC.
References

Table 1. Top10 miRNAs expressed in bone marrow between patients with liver metastasis and non-metastasis

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<th>Rank</th>
<th>miRNAs</th>
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<th>P value</th>
<th>Anti-oncomir or Oncomir</th>
<th>Target</th>
<th>Ref.</th>
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<td>1</td>
<td>miR-340</td>
<td>3.39</td>
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<td>Anti-oncomir</td>
<td>c-Met, MITF(^{a})</td>
<td>24, 25</td>
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<td>2</td>
<td>miR-542-3p</td>
<td>2.91</td>
<td>0.037</td>
<td>Anti-oncomir</td>
<td>Survivin, ILK(^{b})</td>
<td>26, 27</td>
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<td>4</td>
<td>miR-630</td>
<td>2.37</td>
<td>0.035</td>
<td>Anti-oncomir</td>
<td>N.R.(^{c})</td>
<td>28, 29</td>
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<td>6</td>
<td>miR-362-3p</td>
<td>1.78</td>
<td>0.025</td>
<td>Anti-oncomir</td>
<td>E2F1, USF2(^{d}), PTPN1(^{e})</td>
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<td>9</td>
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Increased miRNAs (liver metastasis patients)

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<th>P value</th>
<th>Anti-oncomir or Oncomir</th>
<th>Target</th>
<th>Ref.</th>
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<td>3</td>
<td>miR-222</td>
<td>2.43</td>
<td>0.036</td>
<td>Oncomir</td>
<td>p27, p53 PTEN(^{f}), TIMP3(^{g}), TRPS1(^{h})</td>
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<td>5</td>
<td>miR-151-5p</td>
<td>1.90</td>
<td>0.028</td>
<td>Oncomir</td>
<td>RhoGDIA(^{i})</td>
<td>34</td>
</tr>
<tr>
<td>7</td>
<td>miR-155</td>
<td>1.77</td>
<td>0.040</td>
<td>Oncomir</td>
<td>CDC73(^{j}), TP53BP1(^{k}), SOCS1(^{l})</td>
<td>35-37</td>
</tr>
<tr>
<td>8</td>
<td>miR-501-5p</td>
<td>1.60</td>
<td>0.028</td>
<td>N.R.</td>
<td>N.R.</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>miR-652</td>
<td>1.50</td>
<td>0.049</td>
<td>N.R.</td>
<td>N.R.</td>
<td></td>
</tr>
</tbody>
</table>

NOTE:
Abbreviations: \(^{a}\)MITF, Microphthalmia-associated transcription factor; \(^{b}\)ILK, integrin-linked linase; \(^{c}\)N.R., not reported; \(^{d}\)USF2, upstream transcription factor; \(^{e}\)PTPN1, protein tyrosine phosphatase non receptor type1; \(^{f}\)PTEN, phosphatase and tensin homolog; \(^{g}\)TIMP3, tissue inhibitors of metalloproteinases; \(^{h}\)TRPS1, trichorhinophalangeal syndrome type 1; \(^{i}\)RhoGDIA, Rho GDP dissociation inhibitor (GDI) alpha; \(^{j}\)CDC73, cell division cycle 73; \(^{k}\)TP53BP1, tumor protein p53 binding protein 1; \(^{l}\)SOCS1, suppressor of cytokine signaling 1.
Table 2. Univariate and multivariate analysis for 5-year disease free survival (Cox proportional regression model)

<table>
<thead>
<tr>
<th>Factors</th>
<th>Univariate</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RR</td>
<td>95%CI</td>
</tr>
<tr>
<td>Gender (Male/Female)</td>
<td>1.621</td>
<td>0.739-3.805</td>
</tr>
<tr>
<td>Histroical grade^a (Others/Well-Mod)</td>
<td>1.920</td>
<td>0.455-5.544</td>
</tr>
<tr>
<td>Size (cm) (&gt;3cm/\leq3cm)</td>
<td>2.464</td>
<td>0.854-10.41</td>
</tr>
<tr>
<td>Lymph node metastasis (Present/Absent)</td>
<td>4.145</td>
<td>1.570-14.26</td>
</tr>
<tr>
<td>Venous invasion (Present/Absent)</td>
<td>1.490</td>
<td>0.677-3.427</td>
</tr>
<tr>
<td>miR-340 expression (low/high)</td>
<td>2.637</td>
<td>1.157-6.755</td>
</tr>
<tr>
<td>Serosal invasion (SE-SI/Tis-SS)</td>
<td>4.008</td>
<td>1.817-8.917</td>
</tr>
<tr>
<td>Tumor type (3-4/0-2)</td>
<td>1.656</td>
<td>0.574-6.996</td>
</tr>
<tr>
<td>Tumor site (rectum/colon)</td>
<td>1.235</td>
<td>0.556-2.724</td>
</tr>
</tbody>
</table>

Abbreviation: RR, relative risk; 95% CI, 95% confidence interval

^aHistroical grade, Well, well differentiated adenocarcinoma; Mod, moderately differentiated adenocarcinoma; Others, poorly differentiated and mucinous adenocarcinoma

^bP < 0.05
Figure Legends

Figure 1. Characteristics of CD14−CD45−EpCAM+ cells. (A) Schematic of the immunomagnetic beads-based separation of bone marrow cells using auto MACSTM pro. To obtain the EpCAM+ fraction, CD14+ cells and CD45+ cells were excluded from the whole bone marrow (BM1), then EpCAM− cells were collected using the anti-EpCAM (HEA-125) antibody; this fraction was named BM4 (CD14−CD45−EpCAM+). (B) Transcript expressions in BM4 (epithelial fraction) relative to BM2 (monocyte or macrophage lineage). Compared to BM2, BM4 showed 9.25-fold higher levels in the epithelial marker E-cadherin mRNA, a 0.48-fold change the mesenchymal marker Vimentin mRNA, and no change in N-cadherin level. **P < 0.0001, *P < 0.005, Mann-Whitney U test. (C) Relative expression of CEA and MYC transcripts. Compared to BM2, BM4 showed significantly higher levels of CEA and MYC mRNA (19.5-fold and 2.26-fold, respectively). **P < 0.0001, *P < 0.005, Mann-Whitney U test. (D–E) Immunostaining of BM4 cells using the epithelial cell specific antibodies Ber-EP4 Ab (D) and cytokeratin Ab (E). Tumor cell clusters comprised of individual cells of >10 μm in diameter were clearly visualized. Magnification: ×40. (F) Heat map of the miRNA profile in BM4 cells from CRC patients with liver metastasis and those without metastasis. Using miRNA microarray analysis, we determined the miRNA levels in BM4 cells obtained from twelve non-metastasis patients and seven liver metastasis patients.
Cluster analysis showed 10 miRNAs that were significantly differentially expressed between the two groups with a >1.50-fold change (range, 1.50- to 3.39-fold). The heat map summarizes the expressions of these 10 miRNAs from BM4 in bone marrow cells of 19 patients. Colors range from green to red, corresponding to low to high expression, respectively. Patients No. 1–12 had no distant metastasis and patients No. 13–19 had liver metastasis. miRNAs in italic were decreased and miRNAs in bold were increased in patients with liver metastasis. *P*-values < 0.05, unpaired t test.

Figure 2. *In vitro* inhibitory effects of miR-340 and miR-542-3p on proliferation of CRC cells and miR-340 and miR-542-3p expression in clinical CRC samples.

(A) Pre-miR-340 treatment led to significant growth inhibition in HCT116 and SW480, compared to that of parental cells or cultures treated with pre-miR negative control (*P* < 0.01), n = 4 for each group, Mann-Whitney U test. The results are the mean ± SE of three replicates. The miR-340 expression in pre-miR-340-treated CRC cells was significantly higher than that of pre-miR negative control-treated cells and clinical normal mucosa samples. (B) Similar results were obtained in miR-542-3p. (C) Expression of miR-340 and miR-542-3p in normal colonic mucosa and CRC tissue samples. miR-340 expression was significantly higher in normal mucosa than in CRC tissue (*P* = 0.010). No significant difference was found in miR-542-3p expression (*P* = 0.973). Analyzed samples of 16
CRC patients included 3 stage I, 4 stage II, 5 stage IIIA/B, 1 stage IIIC, and 3 stage IV CRC diseases.

Figure 3. Impact of miR-340 expression on prognosis of CRC patients and systemic delivery of formulated miR-340 to established CRC tumors in vivo. (A) Impact of miR-340 expression on prognosis of patients with colorectal cancer. Seventy-three patients had less than average miR-340 expression levels, and 63 patients had above average miR-340 expression. Kaplan–Meier estimation indicated that high miR-340 expression was significantly associated with better 5-year disease free survival (DFS) and better 5-year overall survival (OS) ($P = 0.023$ and 0.046, respectively). miR-542-3p expression had no effect on 5-DFS or 5-OS ($P = 0.429$, 0.577; data not shown). (B) In therapeutic animal models with HCT116 tumors, tumor growth was significantly inhibited by systemic administration of formulated miR-340 compared to the pre-miR negative control group ($P < 0.05$). Mice were treated on days 6, 9, 12, and 15 with carbonate apatite-formulated pre-miR-340 or pre-miR negative control via tail vein injection. Each shot contained 30 $\mu$g of formulated oligo, with a 40% conjugation rate. miR-340-treated ($n = 7$), miR-NC-treated ($n = 9$), and parent ($n = 8$) for each group, $*P < 0.05$. The miR-340 expression in tumors at 1, 8, and 12h after systemic administration of pre-miR-340 into mice tail vein was significantly higher than that of mice treated with pre-miR negative control ($P < 0.01$). The results are the mean ± SE. $n = 5$ for each group.
(C) Representative image of tumor growth of posterior bilateral flanks in each group after treatment. The arrows indicate tumor location. Left: non-treated mouse, middle: pre-miR negative control-treated mouse, right: miR-340-treated mouse.

Figure 4. c-Met inhibition by miR-340 in vitro and combination survival analysis of c-Met and miR-340. (A) The expression level of c-Met of pre-miR-340-treated HCT116 and SW480 were suppressed to 24.6 and 35.9% of that of negative control miR-treated cells ($P < 0.05$, $n = 3$ for each group). The results are the mean ± SE of two replicates. (B) c-Met expression in the subcutaneous tumors was significantly suppressed at 8, 12, and 24h after systemic administration of miR-340 ($P < 0.01$, $n = 5$ for each group). (C) Combination survival analysis of c-Met and miR-340 expression in 135 patients with CRC. (One of initial 136 samples was used out.) c-Met-high and c-Met-low groups were divided at the median value. Kaplan–Meier curves indicated that miR-340-low/c-Met-high group alone showed a significantly worse DFS compared to miR-340-high/c-Met-low cases ($P = 0.015$).
Figure 1
Figure 2

A

HCT116

SW480

Cell count ($\times 10^6$)

24 h 48 h 72 h

Pre-miR negative control
Pre-miR-340

* $P < 0.01$

B

HCT116

SW480

Cell count ($\times 10^6$)

24 h 48 h 72 h

Pre-miR negative control
Pre-miR-542-3p

* $P < 0.01$

C

miR-340

miR-542-3p

Relative expression

normal mucosa (n = 16) CRC (n = 16)

* $P = 0.010$

Relative expression

normal mucosa (n = 16) CRC (n = 16)

* $P = 0.973$
Figure 3
Molecular Cancer Therapeutics

Decreased miR-340 expression in bone marrow is associated with liver metastasis of colorectal cancer

Hiroshi Takeyama, Hirofumi Yamamoto, Shinya Yamashita, et al.

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